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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 9/127	A1	(11) International Publication Number: WO 94/20073 (43) International Publication Date: 15 September 1994 (15.09.94)
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(54) Title: LIPID-POLYMER CONJUGATES AND LIPOSOMES (57) Abstract A lipid-polymer conjugate for use in forming long-circulating liposomes is disclosed. The conjugate includes a vesicle-forming lipid having covalently attached to its polar head group, one of the polymers: polyvinylpyrrolidone, polyvinylmethylether, polyhydroxypropyl methacrylate, polyhydroxypropylmethacrylamide, polyhydroxyethyl acrylate, polymethacrylamide, polydimethylacrylamide, polymethyloxazoline, polyethyloxazoline, polyhydroxyethyloxazoline, polyhydroxypropyloxazoline, or polyaspartamide. A method for preparing liposomes containing the lipid-polymer conjugate is also disclosed.		

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LIPID-POLYMER CONJUGATES AND LIPOSOMESField of the Invention

The present invention relates to a lipid-polymer
5 conjugate, and to the use thereof in liposomes.

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Background of the Invention

Liposomes have the potential as versatile drug carriers in the bloodstream. Because they contain both an inner aqueous compartment and an outer lipophilic shell, liposomes can be loaded with both water-soluble or lipid-soluble drugs. Since liposomes can be formed of natural biochemical lipid, such as phospholipids and cholesterol, they can be broken down by the body without toxic side effects (Poznansky).

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Until recently, the potential of liposomes as drug carriers has been limited by the rapid clearance of liposomes from the bloodstream. For example,

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conventional liposomes may be largely cleared from the bloodstream within 1-2 hours after intravenous administration (Saba).

A variety of approaches for extending the circulation time of liposomes have been proposed. Two of these have been successful in extending the halflife of liposomes in the bloodstream by periods of up to 40-50 hours. In one approach, described in co-owned Patent No. 4,837,028, liposomes are formulated with the ganglioside G_{M1} and predominantly rigid lipids. In another general approach, disclosed in co-owned Patent No. 5,013,556, liposomes are coated with a layer of polyethylene glycol (PEG) chains.

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Summary of the Invention

It is one general object of the present invention to provide novel lipid-polymer conjugates for use in preparing liposomes having long circulation times in the bloodstream.

It is another object of the invention to provide such long-circulating liposomes.

The invention includes, in one aspect, a lipid-polymer conjugate capable of incorporation into liposomes. The conjugate includes (i) a vesicle-forming lipid having a polar head group, and (ii) covalently attached to the head group, a polymer chain containing a polyvinylpyrrolidone, polyvinylmethylether, polyhydroxypropyl methacrylate, polyhydroxylpropylmethacrylamide, polyhydroxyethyl acrylate, polymethacrylamide, polydimethylacrylamide, polymethyloxazoline, polyethyloxazoline, polyhydroxyethyloxazoline, polyhydroxypropyloxazoline, or polyaspartamide polymer or polymer region. The polymer chain is

characterized, in free form, by a solubility in water of at least 5% (w/v) at room temperature, and a solubility in chloroform, acetonitrile, dimethylformamide, and/or methylene chloride of at least about 0.5% (w/v) at room temperature.

In one preferred embodiment, the polymer chain is a homopolymer of one of the specified polymers, more preferably a homopolymer of polyvinylpyrrolidone, polymethyloxazoline, polyethyloxazoline, or polyhydroxypropylmethacrylamide.

In another embodiment, the polymer is a block copolymer of one of the specified polymers and polyethyleneglycol (PEG).

In a further embodiment, the polymer is a random copolymer formed from vinylpyrrolidone and another monomer, such as vinyl acetate or acrylamide.

The above-listed lipid-polymer conjugates can be end-functionalized to permit attachment of therapeutically active compounds to the conjugate.

In another aspect, the invention includes a method for preparing a liposome characterized by an extended blood circulation time. The method includes adding to vesicle-forming lipids, between 1-30 mole percent of the lipid-polymer conjugate and a pharmaceutical compound to form liposomes containing vesicle-forming lipids, the lipid-polymer conjugate, and the pharmaceutical compound in entrapped form, and sizing the liposomes to a selected size in the size range between about 0.05 to 0.2 microns.

Liposomes prepared in accordance with the present invention have characteristic surface properties. For example, liposome surface charge is shielded. Shielding of charge is measured by a reduction in the electrophoretic mobility of the

liposomes. Reduced mobility reflects a reduction in the zeta potential of the liposomes.

The electrophoretic mobility of liposomes containing the lipid-polymer conjugate is compared to that of the same liposomes where phosphatidylglycerol is substituted for the lipid-polymer conjugate. Both lipid-polymer conjugate and phosphatidylglycerol contribute a negative charge to the liposome surface, so both liposome preparations have the same net surface charge. However, the electrophoretic mobility of liposomes containing the lipid-polymer conjugate is reduced with respect to liposomes containing phosphatidylglycerol.

These and other objects and features of the present invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 illustrates a synthetic reaction scheme for polymerizing vinylpyrrolidone;

Figure 2 illustrates a synthetic reaction scheme for polymerizing 2-methyloxazoline;

Figure 3 illustrates a reaction scheme for polyaspartamide synthesis;

Figure 4 illustrates a synthetic reaction scheme for random copolymerization of vinylpyrrolidone and methyl acrylate monomers;

Figure 5 illustrates a synthetic reaction scheme for a block copolymer with polyvinylpyrrolidone and polyethyleneglycol segments;

Figure 6 illustrates coupling of polyvinylpyrrolidone to a phospholipid by use of N-hydroxysuccinimide; and

Figure 7 illustrates conversion of the terminal hydroxyl group of polymethyloxazoline to a carboxylic acid group, and coupling of the polymer to a phospholipid through this carboxyl group.

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Detailed Description of the Invention

I. Definitions

Unless indicated otherwise, the terms below have the following definitions:

10 "Homopolymers" are polymers which have one monomer in their composition.

"Copolymers" are polymers which have more than one type of monomer in their composition. Copolymers may be block copolymers or random copolymers. Block
15 copolymers contain alternating blocks (segments) of different homopolymers. Random copolymers contain random sequences of two or more monomers.

The term "vesicle-forming lipid" is intended to include any amphipathic lipid having a hydrophobic
20 moiety and a polar head group, and which (a) by itself can form spontaneously into bilayer vesicles in water, as exemplified by phospholipids, or (b) is stably incorporated into lipid bilayers in combination with other amphipathic lipids, with its
25 hydrophobic moiety in contact with the interior, hydrophobic region of the bilayer membrane, and its polar head group moiety oriented toward the exterior, polar surface of the membrane.

A polymer is "soluble" in water if the polymer
30 (either a homopolymer or copolymer) is soluble to at least 5% by weight at room temperature at a polymer size between about 20-150 subunits.

A polymer is "soluble" in a polar organic solvent, which may chloroform, acetonitrile,
35 dimethylformamide, and/or methylene chloride, if the

polymer (either a homopolymer or copolymer) is soluble to at least 0.5% by weight at room temperature, at a polymer size between about 20-150 subunits.

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II. Preparation of Polymer-Lipid Conjugate

The lipid-polymer conjugate of the invention includes (i) a vesicle-forming lipid having a polar head group, and (ii) covalently attached to the head group, a polymer chain having selected solubility properties, as described below. This section describes the preparation of the conjugate.

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A. Vesicle-Forming Lipid

The vesicle-forming lipids used in the lipid-polymer conjugate for forming liposomes of the invention may be selected from a variety of synthetic vesicle-forming lipids or naturally-occurring vesicle-forming lipids. Typically, these lipids may include phospholipids, sphingolipids and sterols.

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An important feature of the vesicle-forming lipid used in the lipid-polymer conjugate is that the lipid contain a chemical group at its polar head group suitable for covalent attachment of a polymer chain. The polar head group may contain, for example, an amine group, hydroxyl group, aldehyde group or a carboxylic acid group.

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Additionally, the vesicle-forming lipid of the lipid-polymer conjugate is selected to achieve a specified degree of fluidity or rigidity, to control the stability of liposomes in serum and to control the rate of release of entrapped drug from liposomes in the blood stream. These lipids may also be selected, in lipid hydrogenation characteristics, to achieve desired liposome preparation properties. It

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is generally the case, for example, that more fluidic lipids are easier to formulate and size by extrusion than more rigid lipid components.

A preferred embodiment of the vesicle-forming lipid of the lipid-polymer conjugate is a lipid having two hydrocarbon chains, typically acyl chains, and a polar head group containing a chemical group. Included in this class are the phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylinositol (PI), and sphingomyelin (SM), where the two hydrocarbon chains are typically between about 14-22 carbon atoms in length, and have varying degrees of unsaturation.

Phosphatidylethanolamine (PE) is an example of a phospholipid which is preferred for the invention since it contains a reactive amino group which is convenient for lipid coupling to polymers. One preferred PE illustrated in the examples is distearyl PE (DSPE).

B. Polymers

The polymer chain in the conjugate is formed from polyvinylpyrrolidone, polyvinylmethylether, polyhydroxypropyl methacrylate, polyhydroxyethyl acrylate, polymethacrylamide, polydimethylacrylamide, polymethyloxazoline, polyethyloxazoline, or polyaspartamide polymers. The polymer chain preferably contains between 20 to 150 monomer subunits, more preferably 40 to 100 monomer subunits.

The chain may be a homopolymer of the selected polymer or a random or block copolymer containing one or more blocks of the selected polymer spaced by blocks or single subunits of a second polymer. The

second polymer may be another of the above-specified polymers or another polymer type, with the limitation that the random or block copolymer meet the following solubility constraints which are important to the properties of the lipid-polymer conjugate in liposomes, as discussed below. Specifically, the copolymer composition is such that the polymer is characterized, in free form (unattached to the lipid moiety), by a solubility in water of at least 5% (w/v) at room temperature, and a solubility in chloroform, acetonitrile, dimethylformamide, and/or methylene chloride of at least about 0.5% (w/v) at room temperature.

Preferred homopolymers and copolymers, and their methods of synthesis will now be considered.

1. Homopolymer Solubility Properties. A preferred homopolymer in the invention is polyvinylpyrrolidone (PVP). This polymer is readily soluble (at least 5% by weight) in cold water, as indicated in Table 1 below (Molyneux). The polymer is also soluble (at least 0.5% by weight) in one or more of the polar solvents chloroform, acetonitrile, dimethylformamide, and/or methylene chloride. The PVP polymers shown in Table 1 have subunit numbers ranging from about 60 (PVP7000) to 3500 (PVP400,000). As defined herein, the specified solubilities in water and a polar organic solvent are determined for a polymer having the desired polymer size of between about 20-150 subunits. Therefore, the solubilities listed in Table 1 for polymers of larger sizes, such as a PVP chain with 3,500 subunits, likely reflect minimum solubility values for polymers of smaller sizes, such as PVP chains with about 20-150 subunits.

Table 1		
PVP10000	water	10 wt%
PVP40000	chloroform	3 wt%
PVP7000	methanol	2 wt%
PVP400000	dimethylformamide	1 wt%
PVP20000	cyclohexanone	0.5 wt%

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10 Considering the solubility properties of other
homopolymers mentioned above,
poly(dimethylacrylamide) is soluble in water and
organic solvents, such as methanol, ethanol, and
dioxane. Polymethacrylamide is soluble in water,
15 methanol, ethylene glycol, and acetone, while it is
insoluble in hydrocarbons and diethyl ether
(Molyneux).

 Polyethyl-and polymethyloxazolines are soluble
in water, and soluble in acetonitrile, chloroform,
20 and dimethylformamide (Molyneux).

 Polyvinylmethylether is soluble in water, and
also in alcohols, amines, esters, aromatic
hydrocarbons, and chlorinated hydrocarbons
(Molyneux).

25 Polyaspartamide is a polymer derived from
aspartic acid and rendered soluble in water by
reaction with ethanolamine which generates hydroxyl
groups along the polymer chain. This polymer is
soluble in water and dimethylformamide (Neri).

30 Polyhydroxypropyl methacrylate and
polyhydroxyethyl acrylate are also soluble in water
and one or more of the specified polar organic
solvents.

Additionally, the homopolymer can be a derivatized cellulose, such as carboxymethylcellulose, hydroxypropylcellulose, or hydroxyethylcellulose. In underivatized cellulose intermolecular hydrogen-bonding results in decreased solubility of cellulose in water. Derivatizing cellulose hydroxyl groups decreases hydrogen-bonding, and as a consequence increases cellulose solubility in water. Derivatives of cellulose usually are formed with varying degrees of substitution at the hydroxyl groups of C-2, C-3, and C-6.

Preferred derivatized celluloses exhibit the following solubilities. For example, hydroxypropylcellulose and hydroxyethylcellulose are soluble in water, chloroform, acetone, pyridine, cyclohexanone and are not soluble in ethanol, diethyl ether and aliphatic hydrocarbons. Cellulose acetate is soluble in water, methylene chloride, chloroform, tetrahydrofuran, among others. It is insoluble in aliphatic hydrocarbons, ethanol, and benzene (Fuchs).

Table 2 shows comparative solubility testing of four polymers: polyethylene glycol (PEG), polymethyloxazoline (poly(MeOz)), polyethyloxazoline (poly(EtOz)), and polyvinylpyrrolidone (PVP). The solubility of each polymer in ten solvents, ranging from 0.1 to 10.2 in polarity index, was determined by visual inspection and at room temperature, that is at about 24°C.

Polymers were rated as soluble in a particular solvent if the polymer dissolved completely at room temperature. Polymer/solvent combinations that did not completely dissolve at room temperature were warmed slightly, and were rated as soluble upon heating if the polymer dissolved. Polymers that did

not completely dissolve in a particular solvent, even upon heating, were rated as insoluble.

As seen in Table 2, none of the polymers are soluble at room temperature in solvents having a polarity index less than 2.8, with the exception of PEG in benzene. However, all of the polymers are soluble in solvents having a polarity index greater than 5.8.

An important feature of the present invention is that the polymer selected for the lipid-polymer conjugate is soluble in an aqueous medium at room temperature and is also soluble in various organic solvents, as demonstrated for the four polymers in Table 2. Similar methods may be used to verify the solubility characteristics of other polymers useful in the invention.

Table 2
Comparative solubility of four polymers in various solvents

Solvent	Polarity Index	PEG	poly (EtOz)	poly (MeOz)	PVP
petroleum ether	0.1	-	-	-	-
benzene	2.7	+	±	-	-
ethyl ether	2.8	-	-	-	-
n-butanol/i-propanol	3.9	-	+	+	+
THF	4.0	±	+	-	-
chloroform	4.1	+		+	+
ethyl acetate	4.4	±	+	-	-
acetonitrile	5.8	+	+	+	+
DMF	6.4	+	+	+	+
water	10.2	+	+	+	+

+ soluble; - insoluble; ± soluble only upon heating

Alternatively, the polymers in the invention may be copolymers which incorporate the above-named monomers, either as block or random copolymers. For

examples, a polymer containing a high percentage of vinylpyrrolidone and another subunit, such as vinylmethylether, methyl acrylate, hydroxypropyl methacrylate, hydroxyethyl acrylate, methacrylamide, and dimethylacrylamide, may be prepared by radical polymerization methods used in PVP synthesis.

Preferred block copolymers with the requisite solubility characteristics are formed to contain one or more alternating blocks of PEG and one of the homopolymers disclosed above. In a preferred embodiment the heteropolymer is a block copolymer consisting of alternating PVP and PEG blocks, or a single blocks of each.

2. Homopolymer Synthesis. Polyvinylpyrrolidone (PVP), an example of an N-vinyl amide polymer, will be discussed in detail as a preferred embodiment of this aspect of the invention. PVP can be synthesized by free radical, anionic, or cationic polymerization of vinyl pyrrolidone (VP) monomers. Preferably, the monomer is polymerized by the free radical route in the presence of a free radical initiator, such as hydrogen peroxide or azobisisobutyronitrile (AIBN).

As described in Example 1 and illustrated in Figure 1, VP monomers are incubated with mercaptoacetic acid (MACA) and AIBN to favor synthesis of PVP with a molecular weight of about 6,000. MACA is used in the polymerization reaction to generate a chemical group, a carboxyl group, at a polymer free end for coupling to a vesicle-forming lipid. Additionally, MACA concentrations are varied to synthesize polymers of a desired length. For example, a 0.2 molar MACA concentration is used to synthesize PVP polymers of a molecular weight of

about 6,000 daltons. MACA also decreases product heterogeneity, and product may not need further purification by size fractionation (Andreani, Veronese). Alternatively, other mercaptanes, such as
5 mercaptoethanol (ME) or mercaptopropanoic acid (MPA), can replace MACA in the polymerization reaction to generate an appropriate terminal group.

A similar free-radical polymerization method is suitable for the synthesis of polyvinylmethylether,
10 polyhydroxypropyl methacrylate, polyhydroxyethyl acrylate, polydimethyl acrylamide, or polymethacrylamide for generating polymers suitable for this invention.

Figure 2 illustrates a synthetic reaction scheme
15 for a polymerization of 2-methyl-oxazoline (MOZ). In this reaction, MOZ polymerization occurs by a cationic polymerization mechanism. MOZ polymerization is initiated by methyl tosylate which catalyzes a ring opening reaction of 2-methyl-
20 oxazoline. The polymerization reaction is propagated by the polymer "live end" which can cause further 2-methyl-oxazoline ring-opening reactions. After the polymerization reaction an aqueous workup generates a hydroxyl group by displacement of the tosylate group
25 at a polymer end (Saegusa). This hydroxyl group is used for polymer attachment to a vesicle-forming lipid. Reaction conditions are discussed in Example 2.

Nucleophilic reagents, other than water, can be
30 used to provide other functional groups at a polymer end. For example, use of a diamine would generate an amine group at the free end. A similar procedure is used for the synthesis of poly(2-ethyl-oxazoline).

Figure 3 illustrates a synthetic reaction scheme
35 for the formation of polyaspartamide. Aspartic acid

is polymerized by heating for 2-4 hours at 200°C to generate polysuccinimide with an average molecular weight of 11,000 (Vegotski). Polysuccinimide is reacted with ethanolamine. This results in ring-opening of succinimide groups of the polymer chain to generate poly(hydroxyethyl-(D,L-aspartamide)). The terminal carboxylic acid groups are coupled to a vesicle-forming lipid after activation of either or both carboxylic acid groups at one polymer end.

Other homopolymer candidates can be synthesized as follows. To generate cellulose derivatives, cellulose is reacted with chloroacetic acid to form carboxymethylcellulose or with ethylene oxide to form hydroxyethylcellulose. To maximize solubility of derivatized celluloses in water, it is usually necessary to partially hydrolyze the fully derivatized cellulose (Kawaguchi, McCormick). In this manner polymers containing between 40 and 100 sugar units can be generated. This is the desired length for purposes of the invention.

3. Random Copolymer Synthesis. Figure 4 shows the formation of a random copolymer of VP and acrylamide (AA) monomers. As described for the polymerization of VP monomers copolymerization reactions are performed in the presence of a free radical initiator and a terminator such as MACA to introduce an end functional group and to decrease product heterogeneity. The ratio of individual monomers in a polymerization reaction mixture is dependent on the reactivity of each monomer to free radical polymerization and to the monomer ratio desired in the polymerization product (Barabas).

VP copolymerizes readily with a wide variety of other monomers, such as ethyl acrylate, methyl

acrylate, methyl methacrylate, maleic anhydride, dimethylaminoethyl methacrylate, acrylamide, methacrylamide, ethylene, vinyl propionate, vinyl caprolactam, and methyl vinyl ketone. The monomers
5 copolymerized with VP preferably possess similar solubility characteristics as does VP, such as methacrylamide monomer.

Alternatively, the monomers copolymerized with VP may possess different solubility characteristics,
10 such as methylmethacrylate monomers. The methylmethacrylate monomer content of the random copolymer is selected so that the polymer product has solubility characteristics similar to those of PVP.

15 4. Block Copolymer Synthesis. Figure 5 illustrates the synthesis of a block copolymer containing a PVP and a PEG block. A short PVP polymer chain prepared as described above can be coupled to a bifunctionalized PEG polymer chain
20 containing terminal amine and carboxylic groups by standard coupling methods to generate an amide linkage (Zalipsky, 1986).

Other block copolymers containing blocks of PEG and blocks of any of the other homopolymers disclosed
25 possessing the requisite solubility properties can be formed in a similar manner by reacting a homopolymer containing a chemical group at one of its ends with a bifunctionalized polymer chain.

Heteropolymers containing more than one
30 alternating block of PEG and any of the disclosed homopolymers can be formed by reacting bifunctionalized PEG chains with bifunctionalized homopolymer chains in the presence of a linking reagent, such as a diisocyanate. The heteropolymer
35 product should possess the requisite solubility

properties. In a preferred embodiment the heteropolymer contains several alternating blocks of PVP and PEG.

5 C. Coupling Methods

In general the covalent attachment of polymers to a vesicle-forming lipid is accomplished by activation of chemical groups at one polymer end prior to reaction with a vesicle-forming lipid. A
10 terminal hydroxyl, amine or carboxyl group may be activated for coupling to the lipid by monofunctional activating agents, such as N-hydroxysuccinimide, ethylchloroformate, DCCD, Woodward's Reagent K, cyanuric acid and trifluoromethanesulfonyl chloride
15 among others. A number of bifunctional crosslinking reagents containing groups with different reactivities, such as some diisocyanates, may also be used to activate polymers prior to coupling to lipid components.

20 A preferred method for activating a PVP polymer for attachment to a phospholipid is illustrated in Figure 6. In this reaction the terminal carboxyl group of the polymer is activated by reaction with N-hydroxysuccinimide. After this activation step the
25 polymer is reacted with an amino group- containing phospholipid, such phosphatidylethanolamine, to generate the polymer derivatized vesicle-forming lipid which is part of the composition of the invention.

30 Methods known to one skilled in art are available for activation of terminal hydroxyl groups (Zalipsky, 1992). In one such method, illustrated in Figure 7, polymethyloxazoline is reacted with succinic anhydride to generate a carboxyl group at a
35 polymer end. The terminal carboxyl group of the

polymer is activated by reaction with N-hydroxysuccinimide. After this activation step the polymer is reacted with an amino group-containing phospholipid, such as phosphatidylethanolamine, forming the desired product.

Most of the polymers described in this invention can be coupled by either of the above described coupling methods. For coupling of derivatized celluloses, the polymers are incubated in the presence of an amine group-containing lipid without any prior activation step. Coupling can occur at the reducing end of the polysaccharide chain by reductive amination.

15 III. Liposome Composition

The polymer-lipid conjugate of the invention is used in preparing liposome compositions designed for use in delivering a drug via the bloodstream. In one embodiment the polymer-lipid conjugate, when incorporated at a mole ratio of preferably 1-30 mole percent in the outer lipid layer of the liposomes, forms a polymer layer which is effective to extend the blood circulation time of the liposomes severalfold over that of the liposomes lacking the polymer-lipid conjugate.

A. Lipid Components

The liposome is composed of underivatized vesicle-forming lipids and polymer-lipid conjugates which have been described above. The underivatized vesicle-forming lipids will form the bulk of the vesicle structure in the liposome.

Generally, these vesicle-forming lipids include any amphipathic lipids having hydrophobic and polar head group moieties, and which (a) can form

spontaneously into bilayer vesicles in water, as exemplified by phospholipids, or (b) are stably incorporated into lipid bilayers, with its hydrophobic moiety in contact with the interior, hydrophobic region of the bilayer membrane, and its polar head group moiety oriented toward the exterior, polar surface of the membrane.

The vesicle-forming lipids of this type are preferably ones having two hydrocarbon chains, typically acyl chains, and a polar head group. Included in this class are the phospholipids, such as phosphatidylcholine (PC), PE, phosphatidic acid (PA), phosphatidylinositol (PI), and sphingomyelin (SM), where the two hydrocarbon chains are typically between about 14-22 carbon atoms in length, and have varying degrees of unsaturation.

The above-described lipids and phospholipids whose acyl chains have a variety of degrees of saturation can be obtained commercially, or prepared according to published methods. Other lipids that can be included in the invention are glycolipids and sterols, such as cholesterol.

The second type of lipid in the liposome composition is the polymer-lipid conjugate described in Section IIA. This polymer-lipid conjugate is included at a molar concentration sufficient to extend the blood circulation time of the liposomes severalfold over that of the liposomes lacking the polymer-lipid conjugate. The lipid conjugate is typically included at 3-10 mole percent, preferably about 5 mole percent.

One preferred embodiment of the polymer-lipid conjugate is a PVP polymer-derivatized distearylphosphatidylethanolamine (PVP-DSPE). The PVP chain is preferably a PVP chain having a

molecular weight between 2,000-17,000 daltons, more preferably between 4,500 and 11,000 daltons. The lipid is preferably a PE, such as DSPE.

Another preferred embodiment of the polymer-lipid conjugate is a polymethyloxazoline-derivatized distearyl-phosphatidylethanolamine (PMOZ-DSPE). The polymethyloxazoline chain is preferably a chain having a molecular weight between 2,000-16,000 daltons, more preferably between 4,000 and 11,000 daltons.

B. Liposome Preparation

The liposomes may be prepared by a variety of techniques, such as those detailed in Szoka et al, 1980. One method for preparing drug-containing liposomes is the reverse phase evaporation method described by Szoka et al and in U.S. Patent No. 4,235,871. In this method, an organic solution of liposome-forming lipids is mixed with a smaller volume of an aqueous medium, and the mixture is dispersed to form a water-in-oil emulsion, preferably using pyrogen-free components. The drug or other pharmaceutical agent to be delivered is added either to the lipid solution, in the case of a lipophilic drug, or to the aqueous medium, in the case of a water-soluble drug.

After removing the lipid solvent by evaporation, the resulting gel is converted to liposomes, with an encapsulation efficiency, for a water-soluble drug, of up to 50%. The reverse phase evaporation vesicles (REVs) have typical average sizes between about 0.2-0.4 microns and are predominantly oligolamellar, that is, contain one or a few lipid bilayer shells. The REVs may be readily sized, as discussed below, by extrusion to give oligolamellar vesicles having a

maximum selected size preferably between about 0.05 to 0.2 microns.

To form MLV's, a mixture of liposome-forming lipids of the type detailed above dissolved in a suitable solvent is evaporated in a vessel to form a thin film, which is then covered by an aqueous medium. The lipid film hydrates to form MLVs, typically with sizes between about 0.1 to 10 microns. Typically, MLVs are sized down to a desired size range of 0.5 or less, and preferably between about 0.05 and 0.2 microns by extrusion.

One effective sizing method for REV's and MLVs involves extruding an aqueous suspension of the liposomes through a polycarbonate membrane having a selected uniform pore size, typically 0.05, 0.08, 0.1, 0.2, or 0.4 microns (Szoka). The pore size of the membrane corresponds roughly to the largest sizes of liposomes produced by extrusion through that membrane, particularly where the preparation is extruded two or more times through the same membrane.

Alternatively, the REV or MLV preparations can be treated to produce small unilamellar vesicles (SUVs) which are characterized by sizes in the 0.04-0.08 micron range. SUVs may be useful, for example, in targeting a tumor tissue which permits selective passage of small particles, typically than about 0.1 micron, through the capillary walls supplying the tumor. As noted above, SUVs may be formed readily from fluid vesicle-forming lipids.

After final sizing, the liposomes can be treated, if necessary, to remove free (non-entrapped) drug. Conventional separation techniques, such as centrifugation, diafiltration, and molecular-sieve chromatography are suitable. The composition can be

sterilized by filtration through a conventional 0.45 micron depth filter.

Although the polymer-lipid conjugate of the invention is preferably included in the lipid components used in forming liposomes, the conjugates may alternatively be incorporated into the outer liposome layer by diffusion into preformed liposomes. Typically, this is done by incubating preformed liposomes in the presence of the conjugate (which can exist in solution in micellar form) until a desired concentration of the conjugate has been taken up in the liposomes. The suspension may additionally contain surfactants, such as deoxycholate, to facilitate diffusion of the conjugate into liposomes. The surfactant can be removed subsequently, e.g., by dialysis.

The liposomes may be prepared to include surface-bound ligand molecules, such as antibodies, which are effective to bind specifically and with high affinity to ligand-binding molecules, such as antigens, which are localized specifically on target cells. As an example, the ligand molecules may be tumor-specific antibodies, for binding to tumor-specific antigens on tumor cells.

A variety of methods for coupling ligands to the surface of liposomes are known. One method includes incorporation of preformed ligand-derivatized lipid components into liposomes. Alternatively, ligands may be coupled to activated ends of polymer chains in a preformed liposome.

C. Surface Shielding Properties

The derivatized lipid-polymer conjugates prepared as above can be further selected for their ability to shield surface charge on liposomes. The

shielding of surface charge can be measured, for example, by changes in the electrophoretic mobility of negatively charged liposomes, according to methods described below.

5 Table 3 shows the surface charge, zeta potential and the distribution ratio between the mononuclear phagocytic system (MPS) and blood for liposomes containing 3 mole percent of the lipid components at the left in the table. Here PC refers to
 10 phosphatidylcholine, PS refers to phosphatidylserine, PG refers to phosphatidylglycerol, G_{Ti} , G_{Dia} , and G_{MI} refer to different gangliosides, and PEG-DSPE refers to distearylphosphatidyl-ethanolamine derivatized by PEG.

15

Table 3			
Lipid	MPS/Blood level (24 hr)	Surface Potential (mV)	
		Surface Charge	Zeta
20 PC	8.6	0	0
PS	188	-6	-8
PG	123	-6	-8
G_{Ti}		-4.6	-17
G_{Dia}		-6	-12
25 G_{MI}	3.0	-3.4	-5
PEG-DSPE	0.7	Equal to PG	-1.3

30 Ratios of liposomes in the mononuclear phagocytic system (MPS) and in the blood are used as a measure of the blood circulation lifetime of the liposomes *in vivo*, where a lower ratio indicates less uptake by the MPS and longer circulation in the bloodstream. The ratios shown in the table were determined for liposomes distribution

24 hours after intravenous administration, for example by the method described in U.S. Patent No. 4,920,016. As seen, all of the formulations except the one containing PEG-DSPE gave MPS/blood ratios significantly above 1.

5 The surface charge values given in the table were calculated by standard methods, such as those described in McDaniel et al., and reflect the surface density of negative charges on the liposomes. It is noted that the surface charge has been determined with respect to
10 selected ionic strength and pH of the liposome in addition to the mole content of the charged lipid components.

 The zeta potential values in the table provide a measure of the apparent charge on the outer surface of
15 the liposomes. These values are determined from the electrophoretic mobility of the liposomes, according to known methods (Woodle). The zeta potential values thus reflect the charge on the liposomes seen by the electric field during electrophoresis. A less negative zeta
20 potential means that the liposomes have a lower apparent surface charge, as determined from a reduced migration rate in an electric field.

 If the zeta potential values are lower than the surface charge values for any liposome formulation, the
25 reduced zeta potential is likely indicative of screening of the surface charge. There is no charge shielding effect observed in liposomes containing PS, PG, G_{T1}, or G_{D1a}. The liposome formulation containing PEG-DSPE, however, shows charge shielding. The zeta potential of -
30 1.3 mV for PEG-DSPE liposomes (Woodle) represents a severalfold drop in negative charge over liposomes containing phosphatidylglycerol (PG) (zeta potential of -8 mV). Liposomes containing either PG or DSPE have the same surface charge density as do PEG-DSPE liposomes, but
35 the charge is not shielded. This charge shielding effect

correlates with the low MPS/blood ratio observed for this formulation.

Liposomes containing G_{MI} also exhibit charge shielding, but not to the same extent as do PEG-DSPE liposomes. G_{MI} contains a negatively charged sialic acid group that extends away from the liposome surface into the aqueous phase. If the sialic group were located on the liposome surface, the surface charge would be expected to be comparable to that of PG or PEG-DSPE liposomes (-6 mV). However, the surface charge value is lower as expected from the location of the negative charge (-3.4 mV).

The zeta potential of G_{MI} , therefore, is reduced (-5 mV) when compared with a value of -6 mV which is the true liposome charge. Also, the zeta potential is lower than for PG liposomes even though the negative charges are closer to the zeta potential determining plane (McLaughlin, McDaniel). The MPS/blood ratio for G_{MI} liposomes is reduced compared to that of PG or PS liposomes, but is not as low as for PEG-DSPE liposomes.

More generally, in accordance with one aspect of the invention, the lipid-polymer conjugate employed in the liposome composition of the invention is preferably one that is effective to shield liposome charge to an extent effective to reduce the electrophoretic mobility of the liposomes with respect to the same liposomes in which a lipid with , such as phosphatidylglycerol, is substituted for the added conjugate. The lipid-polymer conjugate and phosphatidylglycerol both contain a single negative charge at its lipid polar head group and contribute to the surface charge on the liposomes.

IV. Utility

As noted above, the polymer layer formed on liposomes or on other colloidal drug delivery systems by

the lipid-polymer conjugate functions to extend the blood circulation time of the liposomes severalfold. The enhanced blood circulation time, in turn, will allow a variety of therapeutic uses which are not effective in conventional, rapidly cleared liposomes. Some of these new therapeutic uses include:

1. Prolonged delivery of a drug by release from the particles, as they circulate in the bloodstream over an extended time.
2. Treatment of solid tumors. The long circulation time allows the particles to reach a tumor targeting site via the bloodstream, and to extravasate into the tumor region.
3. Treatment of infection or inflammation. As above, the long circulation time allows the particles to reach a site of infection or inflammation, via the bloodstream, and to extravasate into the region of infection.

A critical feature of the polymer layer on the liposome, as indicated above, is that the polymers forming the layer are soluble in an aqueous medium, but also soluble in one or more of a variety of polar organic solvents. Polymer solubility in water permits the polymer chains to extend away from the liposome surface out into the aqueous shell surrounding the liposome, and to effectively "fill" the aqueous shell. Polymer solubility in a variety of organic solvents and water suggests that the polymer chains are conformationally flexible, and thus are able to create a uniform local concentration of polymer chains around the outer surface of the liposomes forming an effective barrier to interactions between the liposome surfaces and blood components involved in liposome uptake from the blood.

The following examples illustrate methods of preparing lipid-polymer conjugates which can be incorporated in liposome compositions to enhance liposome circulation times in the bloodstream. The examples are intended to illustrate specific lipid-polymer conjugate preparations, but are in no way intended to limit the scope thereof.

EXAMPLE 1

Preparation of PVP-DSPE Conjugate

The polymerization of PVP, and attachment of PVP to DSPE described in this example is illustrated in Figure 1.

A. PVP Polymerization

A mixture of 25 g (0.23 mole) of N-vinyl-2-pyrrolidone, with 0.7 ml (10 mmole) mercaptoacetic acid (in a 70% aqueous solution) and 125 mg (0.76 mmole) of AIBN is diluted to 50 ml with methanol and kept at 60°C with stirring under a nitrogen atmosphere. After 24 hours, 100 ml methylene chloride is added. The solution is dried over anhydrous sodium sulfate, filtered and poured with stirring into an excess of dry ether. The polymer is purified by dissolving in methylene chloride and reprecipitating into an excess of dry ether.

Product is further purified by size fractionation using a Bio Gel P60 column (5 × 50 cm) and water as the mobile phase. PVP polymers with a molecular weight of approximately 6,000 daltons (about 53 monomer units) are isolated.

B. PVP Attachment to DSPE

To activate the carboxylic acid of PVP for coupling to the amine group of DSPE the following protocol is utilized. To a solution of PVP6000, 10 g, 1.7 mmole in

50 ml of N,N-dimethylformamide (DMF) cooled to 10°C, and 0.575 g, 5 mmole of N-hydroxysuccinimide in DMF, and dicyclohexyl carbodiimide (1.032 g, 4.6 mmole) in DMF, are added. The solution is stirred overnight and the temperature is allowed to increase to room temperature. After removal of the precipitated dicyclohexylurea by filtration, the solution is concentrated to dryness under high vacuum. The residue is taken up in 50 ml of methylene chloride and the solution added dropwise to stirred diethyl ether (500 ml). The white precipitate is recovered by filtration and reprecipitated twice from methylene chloride/diethyl ether.

For attachment of PVP6000 to the polar head group of DSPE, to a chloroform solution (10 ml) of N-hydroxysuccinimide-ester PVP (0.8 mmole) is added DSPE (0.52 g, 0.70 mmole) TEA (0.2 ml, 1.4 mmole) to the reaction mixture. The mixture is maintained in an oil bath heated to 40-45°C for 2 hours. The formation of product is confirmed by TLC on silica plates (chloroform/methanol/water/concentrated ammonium hydroxide, (21.5/70/8/0.5)).

EXAMPLE 2

Preparation of Polymethyloxazoline-DSPE Conjugate

The polymerization of 2-methyl-2-oxazoline, and attachment of the polymer to DSPE described in this example is illustrated in Figure 2.

A. Polymerization of 2-methyl-2-oxazoline

Cationic polymerization of 2-methyl-2-oxazoline (MOZ) is carried out by using methyltosylate (MET) as an initiator. To MOZ (3.2 mmole) is added MET (0.07 mmole) in 1 ml acetonitrile. The reaction proceeds for 5 hours at 80°C. The polymerization product is precipitated two times with diethyl ether. The polymerization product is

purified by sizing chromatography to isolate polymer species with about 30 to 60 monomer units. This corresponds to a molecular weight of about 2000 to 4000.

The product is dissolved in water to displace a
5 tosylate group from one of the polymer ends.

B. Attachment of Polymethyloxazoline to DSPE

The terminal hydroxyl group can be converted to a carboxyl group by reaction with succinic anhydride prior
10 to polymethyloxazoline attachment to DSPE.
Polymethyloxazoline (10 mmole) and succinic anhydride (11 mmole) are mixed in 1,2-dichloroethane containing pyridine (10 mmole). The mixture is refluxed under nitrogen for four hours. After filtration and
15 evaporation of the solvent, the residue is dissolved in methylene chloride and precipitated twice by addition of diethyl ether. The polymer attachment to DSPE is performed as described for PVP.

20

EXAMPLE 3

Polyaspartamide Synthesis

Aspartic acid (100 g) is polymerized by heating for 2 hours at 200°C in an open tube. This polymerization reaction results in a polymer of 11,000 daltons. The
25 polymer is dissolved in N,N-dimethylformamide and the solution is poured into a beaker containing 1 liter of water. A flaky precipitate is formed which is filtered, rinsed with water until neutrality, and dried in an oven at 110°C for 24 hours.

30

Poly(D,L-succinimide) (30 g) is dissolved in dimethylformamide. Ethanolamine (45 ml) is added drop by drop and the solution is cooled in an ice bath to keep the temperature at 25-30°C. The mixture is stirred for 2 hours and then neutralized with glacial acetic acid,
35 diluted with water, dialyzed and lyophilized (Neri).

The single amino group of the polymer is used for the selective conjugation with lipid derivatives. For example, by reductive amination with periodate oxidized phosphatidylglycerol or phosphatidylinositol.

5

Example 4

Preparation of VP/acrylamide-DSPE Conjugate

A. Polymerization of VP with Acrylamide Monomers

The VP/acrylamide copolymer is prepared in a similar
10 fashion as described for the PVP homopolymer. N-vinylpyrrolidone (60 mmole) and vinyl acetate (67 mmole) with 0.7 ml (10 mmole) mercaptoacetic acid (in a 70% aqueous solution) and 125 mg (0.76 mmole) of AIBN is diluted to 50 ml with methanol and kept at 60°C with
15 stirring under a nitrogen atmosphere. After 24 hours, 100 ml methylene chloride is added. The solution is dried over anhydrous sodium sulfate, filtered and poured with stirring into an excess of dry ether. The polymer is purified by dissolving in methylene chloride and
20 reprecipitating into an excess of dry ether.

Product is further purified by size fractionation using a Bio Gel P60 column (5 × 50 cm) and water as the mobile phase. PVP/AA copolymers with a molecular weight of approximately 6,000 daltons (about 53 monomer units)
25 are isolated.

B. Attachment of PVP/AA Polymer Product to DSPE

The polymer is coupled to the vesicle-forming lipid by activating the polymer carboxyl group with N-
30 hydroxysuccinimide prior to the addition of DSPE as described for Example 1.

EXAMPLE 5Preparation of PEG/PVP Block Copolymer-DSPE ConjugateA. Preparation of PEG/PVP Block Copolymer

A PVP chain containing a terminal carboxyl group is described in Example 1. Since the desired PVP product is to have an average molecular weight of about 3,000 instead of about 6,000 the concentration of MACA is increased from 0.2 M to 0.4 M. PEG chains with an average molecular weight of 2,000, each with one terminal amine and carboxyl group can be synthesized (Zalipsky). The two polymer segments are then coupled by first activating the PVP carboxyl group with N-hydroxysuccinimide ester and then reacting the activated carboxyl group with the amine group of PEG.

To activate the carboxylic acid of PVP for coupling to the amine group of PEG the following protocol is utilized. To a solution of PVP3000, 1 g in 10 ml of N,N-dimethylformamide (DMF) cooled to 10°C, and equimolar amounts of N-hydroxysuccinimide in DMF, and dicyclohexyl carbodiimide in DMF, are added dropwise. The solution is stirred overnight and the temperature is allowed to increase to room temperature. After removal of the precipitate dicyclohexylurea by filtration, the solution is concentrate to dryness under high vacuum. The residue is taken up in 5 ml of methylene chloride and the solution added dropwise to stirred diethyl ether (100 ml). The white precipitate is recovered by filtration and reprecipitated twice from methylene chloride/diethyl ether.

For attachment of PVP3000 to PEG, to a methylene chloride solution of N-hydroxysuccinimide ester-terminated PVP (2.1 g, 0.70 mmole) is added the omega-amino acid derivative of PEG (1.4 g, 0.70 mmole) in 5 ml methylene chloride followed by TEA (0.2 ml, 1.4 mmole). The reaction mixture is stirred at 25 °C for 22 hours.

The product is precipitated from the methylene chloride solution by addition of diethyl ether. This step is repeated several times.

5 B. PVP-PEG Block Copolymer Attachment to DSPE

To activate the carboxylic acid of PVP-PEG copolymer for coupling to the amine group of DSPE the following protocol is utilized. To a solution of PVP-PEG (MW=5000, 1 g, 0.2 mmole) in 4 ml of N,N-dimethylformamide (DMF) cooled to 10°C, and N-hydroxysuccinimide (30 mg, 0.26 mmole) in DMF (1 ml), and dicyclohexyl carbodiimide (59 mg, 0.26 mmole) in DMF (1 ml), are added. The solution is stirred overnight and the temperature is allowed to increase to room temperature. After removal of the precipitated dicyclohexylurea by filtration, the solution is concentrated under high vacuum. The residue is taken up in 5 ml of methylene chloride and the solution added dropwise to stirred diethyl ether (100 ml). The white precipitate is recovered by filtration and reprecipitated twice from methylene chloride/diethyl ether.

For attachment of PVP-PEG (MW=5000) to the polar head group of DSPE, to the methylene chloride solution of N-hydroxysuccinimide ester terminated PVP-PEG copolymer (3.5 g, 0.70 mmole) is added is added DSPE (0.70 mmole) in 2 ml chloroform and TEA (0.2 ml, 1.4 mmole) are added to the reaction mixture. The mixture maintained on an oil bath heated to 70-75°C for 2 hours. The formation of product is confirmed by TLC on silica plates (chloroform/methanol/water/concentrated ammonium hydroxide, (21.5/70/8/0.5).

Although the invention has been described and illustrated with respect to particular derivatized lipid compositions, it will be apparent that a variety of modifications and changes may be made without departing from the invention.

IT IS CLAIMED:

1. A lipid-polymer conjugate comprising
a vesicle-forming lipid having a hydrophobic
5 moiety and a polar head group, and
covalently attached to the head group, a
polymer chain containing a polymer selected from the
group consisting of polyvinylpyrrolidone,
polyvinylmethylether, polyhydroxypropyl methacrylamide,
10 polyhydroxypropyl methacrylate, polyhydroxyethyl
acrylate, polymethacrylamide, polydimethylacrylamide,
polymethyloxazoline, polyethyloxazoline,
polyhydroxyethyloxazoline, polyhydroxypropyloxazoline,
and polyaspartamide,
15 said polymer chain being characterized, in free
form, by a solubility in water of at least 5% at room
temperature, and a solubility in an organic solvent
selected from the group consisting of chloroform,
acetonitrile, dimethylformamide, and methylene chloride,
20 of at least about 0.5% at room temperature.

2. The conjugate of claim 1, wherein the polymer
chain is a homopolymer of one from the group consisting
of polyvinylpyrrolidone, polyvinylmethylether,
25 polyhydroxypropyl methacrylamide, polyhydroxypropyl
methacrylate, polyhydroxyethyl acrylate,
polymethacrylamide, polydimethylacrylamide,
polymethyloxazoline, polyethyloxazoline,
polyhydroxyethyloxazoline, polyhydroxypropyloxazoline,
30 and polyaspartamide.

3. The conjugate of claim 1, wherein the polymer
chains is a block or random copolymer of polymers
selected from the groups consisting of
35 polyvinylpyrrolidone, polyvinylmethylether,

polyhydroxypropyl methacrylate, polyhydroxyethyl
acrylate, polymethacrylamide, and polydimethylacrylamide.

4. The conjugate of claim 1, wherein the polymer
5 chain is a block or random copolymers of polymers
selected from the group consisting of
polymethyloxazoline, polyethyloxazoline,
polyhydroxyethyloxazoline, and
polyhydroxypropyloxazoline.

10

5. The conjugate of claim 1, wherein the polymer
chain is a block copolymer of a polymer selected from the
group consisting of polyvinylpyrrolidone,
polyvinylmethylether, polymethacrylamide,
15 polydimethylacrylamide, polymethyloxazoline, and
polyethyloxazoline, in combination with polyethylene
glycol.

6. The conjugate of claim 1, wherein the polymer
20 is a homopolymer of polyvinylpyrrolidone.

7. The conjugate of claim 1, wherein the polymer
is a homopolymer selected from the group consisting of
polymethyloxazoline and polyethyloxazoline.

25

8. The conjugate of claim 1, wherein the polymer
chain has degree of polymerization between about 20 to
150.

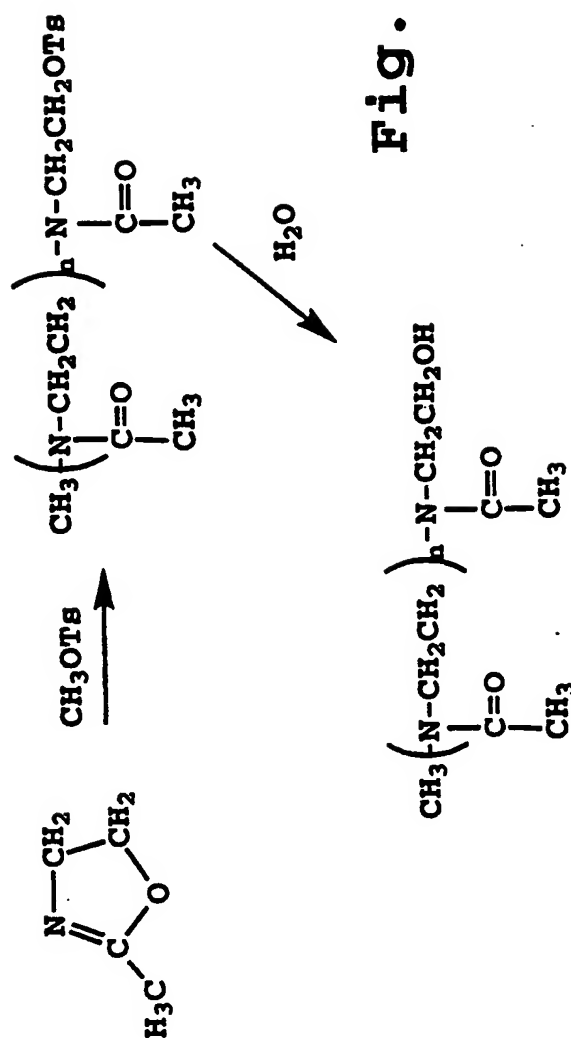
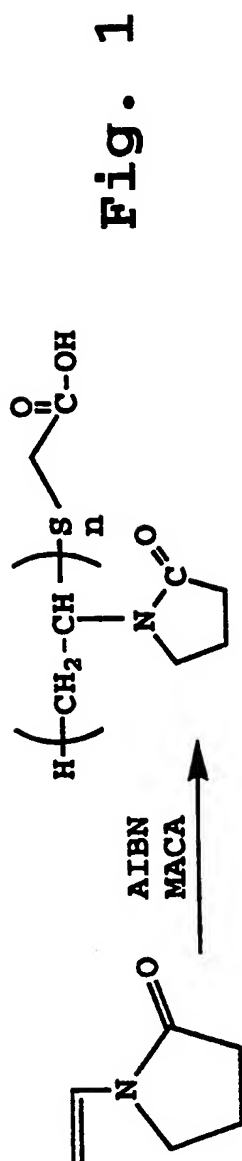
9. The conjugate of claim 1, wherein the vesicle-
30 forming lipid is a phospholipid.

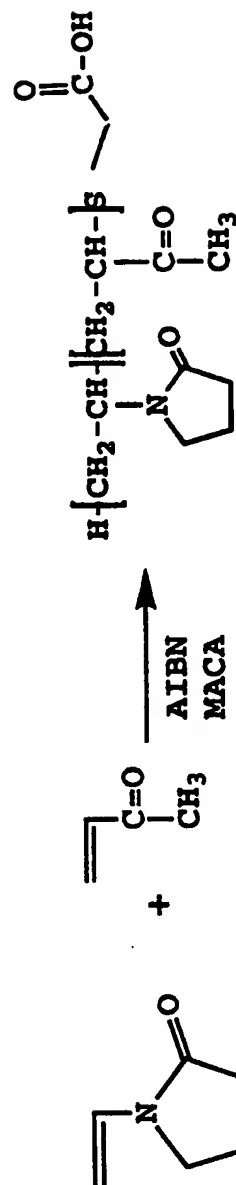
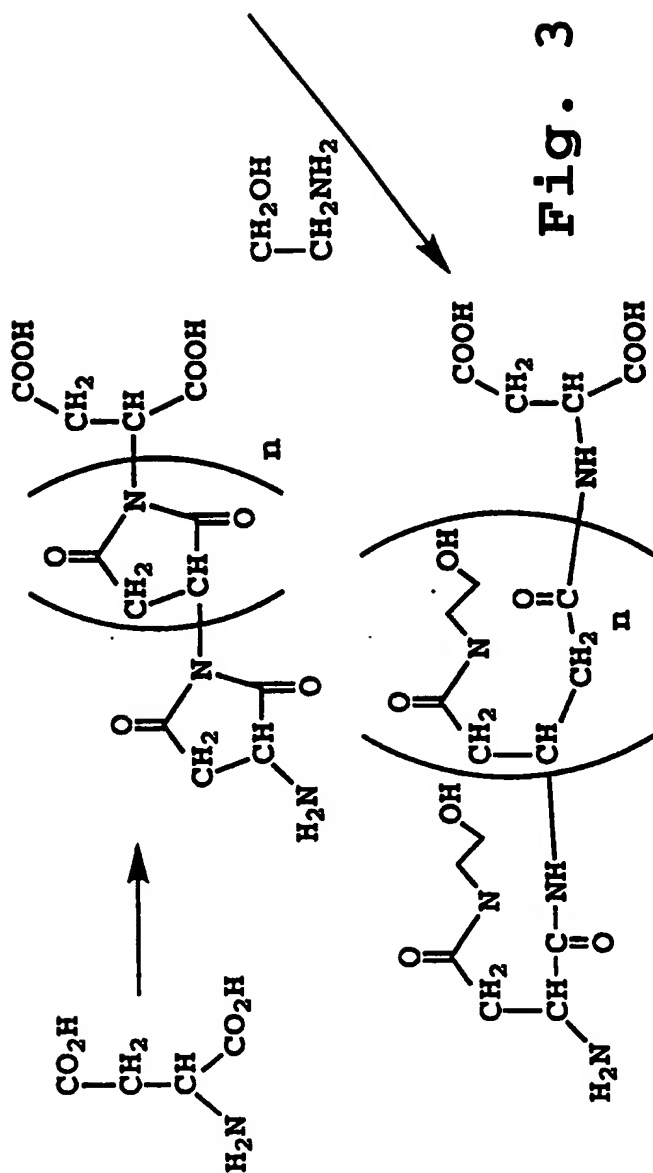
10. The conjugate of claim 9, wherein the vesicle-
forming lipid is phosphatidylethanolamine.

35

11. A method of preparing liposomes characterized by an extended blood circulation time, comprising adding to vesicle-forming lipids, between 1-30 mole percent of a lipid-polymer conjugate having a hydrophobic moiety and a polar head group, and covalently attached to the head group, a polymer chain containing a polymer selected from the group consisting of polyvinylpyrrolidone, polyvinylmethylether, polyhydroxypropyl methacrylate, polyhydroxypropylmethacrylamide, polyhydroxyethyl acrylate, polymethacrylamide, polydimethylacrylamide, polymethyloxazoline, polyethyloxazoline, polyhydroxyethyloxazoline, polyhydroxypropyloxazoline, and polyaspartamide, forming liposomes containing said vesicle-forming lipids and lipid-polymer conjugate, and containing the pharmaceutical compound in entrapped form, and sizing the liposomes to a selected size in the size range between about 0.05 to 0.5 microns, where the added conjugate is effective to extend the circulation time of the liposomes severalfold over that of the same liposomes prepared in the absence of said conjugate.
12. The method of claim 11, wherein the added lipid-polymer conjugate is effective to reduce the electrophoretic mobility of the liposomes with respect to the same liposomes in which phosphatidylglycerol is substituted for the added conjugate.

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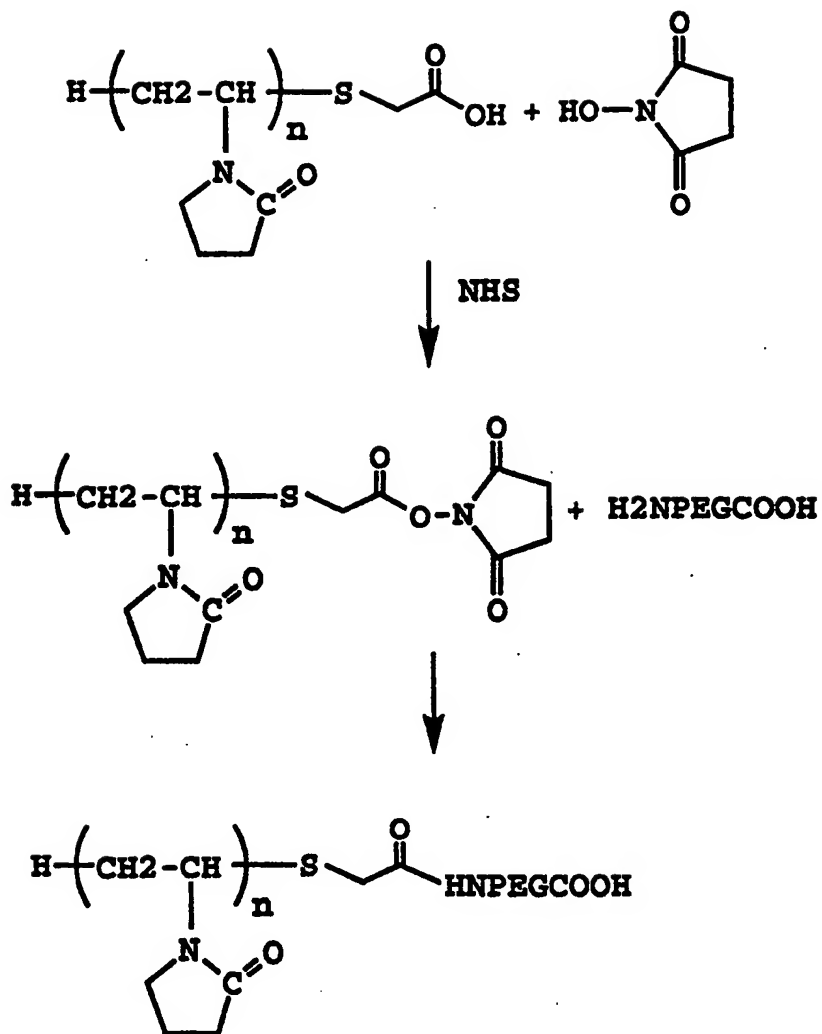


Fig. 5

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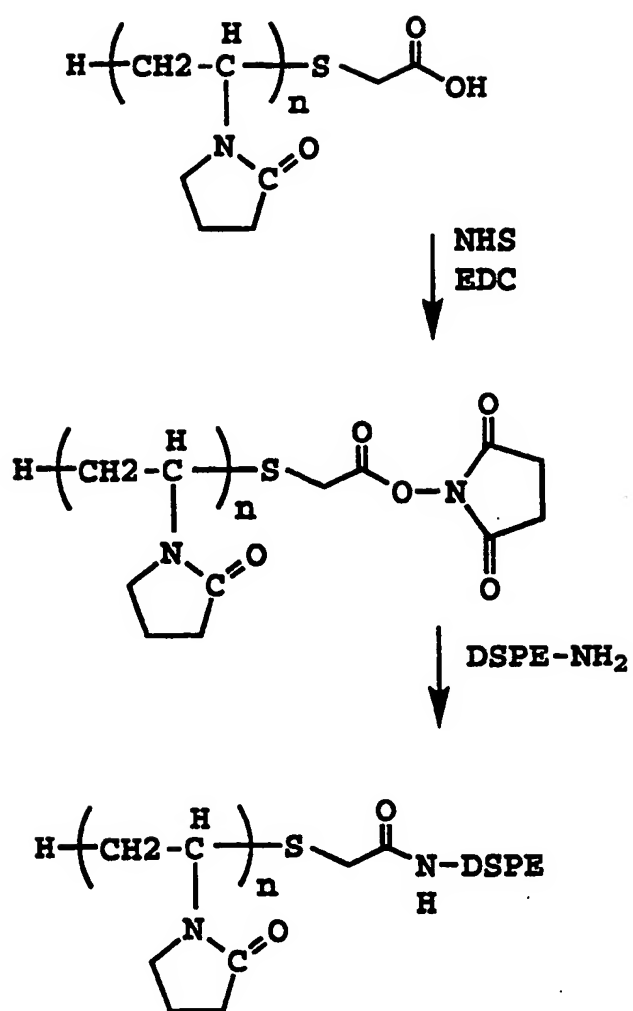


Fig. 6

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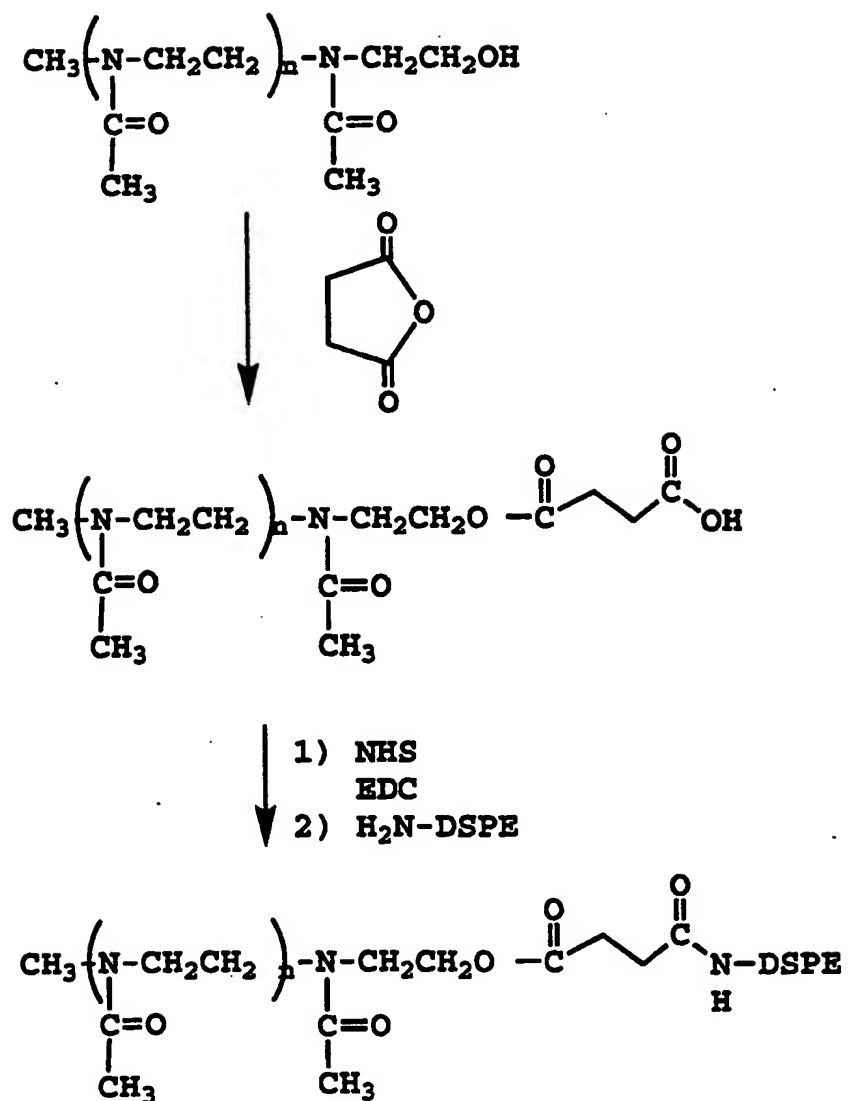


Fig. 7

INTERNATIONAL SEARCH REPORT

Intern. Application No
PCT/US 94/02271

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K9/127

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,91 05545 (LIPOSOSOME TECHNOLOGY) 2 May 1991 see the whole document	1-12
A	& US,A,5 013 556 (MARTIN ET AL) cited in the application ---	
A	DATABASE WPI Week 9217, Derwent Publications Ltd., London, GB; AN 92-138668 & JP,A,4 082 893 (FUJI PHOTO FILM KK) 16 March 1992 see abstract --- -/--	1-12



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Date of the actual completion of the international search

7 July 1994

Date of mailing of the international search report

12.07.94

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p> DATABASE WPI Week 9251, Derwent Publications Ltd., London, GB; AN 92-418589 & JP,A,4 312 535 (TERUMO CORP) 4 November 1992 see abstract ----- </p>	1-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

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